Molecular Biology of the Cell
Vol. 7, 1535–1546, October 1996

Cloning and Functional Characterization of Mammalian Homologues of the COPII Component Sec23

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Submitted April 23, 1996; Accepted July 12, 1996
Monitoring Editor: Suzanne R. Pfeffer

We screened a human cDNA library with a probe derived from a partial *SEC23* mouse homologue and isolated two different cDNA clones (hSec23A and hSec23B) encoding proteins of a predicted molecular mass of 85 kDa. hSec23Ap and hSec23Bp were 85% identical and shared 48% identity with the yeast Sec23p. Affinity-purified anti-hSec23A recognized a protein of ~85 kDa on immunoblots of human, mouse, and rat cell extracts but did not recognize yeast Sec23p. Cytosolic hSec23Ap migrated with an apparent molecular weight of 350 kDa on a gel filtration column, suggesting that it is part of a protein complex. By immunoelectron microscopy, hSec23Ap was found essentially in the ribosome-free transitional face of the endoplasmic reticulum (ER) and associated vesicles. hSec23Ap is a functional homologue of the yeast Sec23p as the hSec23A isoform complemented the temperature sensitivity of the *Saccharomyces cerevisiae sec23-1* mutation at a restrictive temperature of 34°C. RNase protection assays indicated that both hSec23 isoforms are coexpressed in various human tissues, although at a variable ratio. Our data demonstrate that hSec23Ap is the functional human counterpart of the yeast COPII component Sec23p and suggest that it plays a similar role in mammalian protein export from the ER. The exact function of hSec23Bp remains to be determined.

INTRODUCTION

In vitro reconstitution of protein transport between successive intracellular compartments isolated from both mammalian and yeast cells has resulted in the molecular characterization of two distinct coat proteins, COPI and COPII, that are responsible for the formation of vesicles from donor membranes (Rothman and Orci, 1992; Rothman, 1994; Schekman and Orci, 1996).

COPI was identified by the isolation of proteins that mediate vesicle budding within the mammalian Golgi apparatus (Balch *et al.*, 1984; Orci *et al.*, 1986, 1989, 1993b). Upon the binding of ARF-GTP to a putative receptor present on the Golgi membrane coatomer, a 700-kDa protein complex is recruited to form a coated bud that separates from the donor membrane in a process requiring acyl-CoA (Orci *et al.*, 1993a; Ostermann *et al.*, 1993). Coat proteins are shed after ARF hydrolyzes guanosine 5′-triphosphate (GTP), enabling the fusion of the vesicle with an acceptor membrane (Tanigawa *et al.*, 1993).

In mammalian cells, coatomer is localized at the cis-Golgi and the transitional area between the ER and cis-Golgi network (Oprins *et al.*, 1993; Duden *et al.*, 1994) as well as to specialized coatomer-enriched endoplasmic reticulum (ER) structures (CRER; Orci *et al.*, 1994). In addition, COPI may be involved in both forward and retrograde protein trafficking between the ER and the Golgi (Pepperkok *et al.*, 1993; Dascher and Balch, 1994; Letourneur *et al.*, 1994; Pelham, 1994; Zhang *et al.*, 1994; Aridor *et al.*, 1995; Bednarek *et al.*, 1995).

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The analysis of temperature-sensitive secretion mutants in yeast has identified another set of genes involved in ER-derived vesicle biogenesis (*SEC12, SEC13, SEC16, SEC23*; Kaiser and Schekman, 1990). In vitro reconstitution of ER-derived vesicle formation with purified cytosolic components requires five different proteins comprising a novel coat called COPII (Barlowe *et al.*, 1994). The recruitment to the ER membrane of the Sec23/24 complex and Sec13/31 complex is initiated by the binding of Sar1p to the membranes, which is facilitated by the transmembrane protein Sec12p, a nucleotide dissociation factor that stimulates the formation of Sar1p-GTP (Hicke *et al.*, 1992; Barlowe *et al.*, 1993; Barlowe and Schekman, 1993; Salama *et al.*, 1993; Yoshihisa *et al.*, 1993). The uncoating of the vesicles relies on Sar1p GTP hydrolysis, a necessary step in targeting and fusion of the vesicles to the acceptor compartment (Barlowe *et al.*, 1994). Homologues of some COPII components (Sar1p, Sec13p, Sec12p) have been identified in plants and mammals (Orci *et al.*, 1991; d'Enfert *et al.*, 1992; Kuge *et al.*, 1994). In mammalian cells, Sec23p, Sar1p, and Sec13p have been immunolocalized to the smooth transitional zone between the ER and cis-Golgi network (Orci *et al.*, 1991; Kuge *et al.*, 1994; Shaywitz *et al.*, 1995).

In yeast, COPI and COPII-coated vesicles were found to bud directly from the ER (Bednarek *et al.*, 1995). In mammalian semi-intact cells it was proposed that COPII and COPI acted sequentially and in a coupled manner to generate transport vesicles from the ER (Aridor *et al.*, 1995). Thus, more work is needed to understand the respective role of COPI and COPII components in vesicular transport in mammalian cells, and this will require a careful in vitro reconstitution of ER export with the use of purified COPI and COPII components. Whereas the mammalian homologues of yeast Sar1p and Sec13 have been cloned and their role in ER to Golgi transport has been partially investigated, mammalian Sec23 was not characterized. We report here the molecular cloning and functional characterization of two closely related human cDNAs coding for proteins that share significant sequence homology with the yeast Sec23p.

MATERIALS AND METHODS

Reagents, Cell Cultures, and General Methods

Biochemicals were purchased from Merck (Darmstadt, Germany), if not otherwise stated; T3 RNA polymerase, rNTPs, and Pfu DNA polymerase were obtained from Promega (Madison, WI); restriction enzymes, ligase, and calf intestine phosphatase were obtained from New England Biolabs (Beverly, MA); pYES2 and pRSETc vectors were obtained from Invitrogen (San Diego, CA); pBluescript KS (pBS-KS) was obtained from Promega; anti-rabbit Ig-HRP, ECL reagents kit, ^{32}P-UTP, and ^{35}S-ATP were obtained from Amersham (Arlington Heights, IL); Trans^{35}S-Label™ was purchased from ICN Biochemicals (Costa Mesa, CA); monoclonal anti-HA 12CA5 was purchased from Babco (Richmond, CA); T7 DNA sequencing kit and Superdex 200 were purchased from Pharmacia (Piscataway, NJ). Anti-β-COP polyclonal antibody was kindly provided by T. Kreis (University of Geneva, Switzerland).

Mammalian cells were cultured on their appropriate medium supplemented with 10% fetal calf serum at 5% CO_2. Yeast cultures and manipulations were done according to Guthrie and Fink (1991), and, unless stated otherwise, molecular biology procedures were conducted as in Ausubel *et al.* (1992).

Yeast strains included *Saccharomyces cerevisiae* RSY620 (*MAT a, PEP4::TRP1, ade2-1, his3-11, 15, leu2-3-112, trp1-Δ1, ura3-1*) and RSY 868 (*MAT α, sec23-1, his3-11, 15, leu2-3-112, trp1-Δ1, ura3-1, GAL2*); *Escherichia coli* strains were DH5α and BL21/DE3-LysS.

Cloning Procedures

On the basis of the mouse cDNA sequence *Msec23*, several primers were designed to amplify different portions of cDNA coding for the mammalian homologue of Sec23. The sequence of the primers that gave the best amplification products is as follows: sense primer P1, 5′-GATCTAGGAATTCGTTGGCTAATCGAGCTGCTAC-3′, with a 5′ *Eco*RI cloning site; and antisense primer P2, 5′-ACGTAAGCTTA-CATAAACTGAGGATAAAGAG-3′, with a *Hin*dIII cloning site. Amplification was performed on a Perkin–Helmer thermocycler with Pfu DNA polymerase as follows. The first round of amplification consisted of three cycles at an annealing temperature of 54°C for 3 min (elongation at 72°C for 2.5 min and denaturation for 1 min at 94°C); the second round consisted of three cycles at an annealing temperature of 50°C, followed by 25 cycles with annealing at 46°C. Samples were analyzed on agarose gels, and bands at the expected size were excised and the agarose piece incubated in 100 μl of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). Two microliters of the supernatant was submitted to 30 cycles of amplification at an annealing temperature of 46°C. We used as templates cDNA libraries from the AtT20 mouse cell line (kindly provided by H.-P. Moore, University of California, Berkeley, CA) as well as a human B cell cDNA library included in a yeast expression vector (kindly provided by S. Elledge, Baylor College of Medicine, Houston, TX). Fragments of the expected 800-base pair (bp) size were obtained from both the mouse and human templates. The polymerase chain reaction (PCR) fragments obtained were subcloned into pBS-KS by using the *Eco*RI and *Hin*dIII sites and sequenced at its extremities. The nucleotide sequence of the human fragment was 85% identical to the mouse *Msec23* cDNA of Wadhawa *et al.* (1993), indicating that we most likely amplified a fragment of the human *Msec23* homologue, which we named phSec23-3′. We obtained a 5′ fragment of the human cDNA coding for Sec23 by amplifying an aliquot of mRNA from HepG2 cells that was reverse transcribed with Avian Myeloblastosis Virus (AMV) reverse transcriptase with primer P3 (5′-TGTATC-CTCCAGTAAGGT-3). This primer was derived from the human sequence cloned previously. Amplification was done with the antisense primer P4 (5′-AATTGGATCCTGTTTACACCTCTGAAG-GAG-3′) and P3 as described above. The PCR product was cloned into pBS-KS with the *Eco*RV and *Bam*HI and named phSec23-5′.

The phSec23-5′ fragment was used as a probe to screen a size-selected human B lymphocyte cDNA library. Hybridization was done for 24 h at 60°C in 5× SSC, 5× Denhardt's solution, 0.1% SDS, 100 μg/ml herring sperm DNA, and 1 mg/ml yeast tRNA. Membranes were washed twice in 2× SSC, 0.5% SDS at 50°C for 30 min, and twice in 1× SSC. Independent clones (800′000) were screened and 18 positive colonies isolated. After restriction digestion, one clone was selected and sequenced in both directions. This clone was named phSec23A. The identification of hSec23B was performed by screening the same plating of the library with a 25-mer oligonucleotide. This probe was derived from the sequence of the EST65274, which was recovered from the database as a putative isoform of the hSec23A clone (primer EST65274, 5′-TTTATGCTTGTGCCCTTGAT-CAAAC-3′). The ^{32}P-labeled oligonucleotide was hybridized for 24 h at 50°C in 5× SSPE (1× SSPE: 0.15 M NaCl and 10 mM NaH_2PO_4, pH 7.4), 5× Denhardt's solution, 0.1% SDS, 200 μg/ml

herring sperm DNA, and 200 μg/ml yeast tRNA; the membranes were washed twice in 6× SSC, 0.1% SDS at 50°C for 20 min and twice in 4× SSC, 0.1% SDS at 50°C for 25 min. The screen detected 24 positive colonies. At restriction digestion, one clone was selected and sequenced. This clone was 2.8 kb in length and was named phSec23B.

The HA epitope was introduced at the N terminus of hSec23B by PCR. An oligonucleotide was designed that contained at its 5′ terminus the *Eco*RI restriction site, a translation initiation consensus before the ATG, the sequence coding for the HA epitope (YPYDVPDYA), and 14 nucleotides corresponding to the N terminus of hSec23B: 5′-GCGAATTCTCGAGCCACCATGTACCCATACGACGTCCCAGACTACGTCCCTAGGATGGCGACATACCTGGAG-3′. The reverse primer was 5′-CCTCCAGGCATGTGTCAAC-3′. The amplified fragment was subcloned into phSec23B with *Eco*RI and PflMI sites. The amplified fragment was sequenced and found to be correct. For expression in yeast, the tagged construct was then inserted into pYES2 with *Eco*RI and *Xba*I restriction sites.

Antibody Production

Antibodies against hSec23Ap were prepared with a recombinant N-terminal portion of hSec23A encoded by the plasmid phSec23-5′. The *Bam*HI site introduced into the primer P4 enabled the subcloning of the PCR product into the bacterial expression vector pRSETc in frame with the N-terminal histidine tag of the vector. This construction resulted in a sequence that encoded a protein having at its N terminus a stretch of six histidines followed by a fragment of hSec23Ap starting at amino acid 41 of the human hSec23A sequence and extending to amino acid 361. When this construct was introduced into the *E. coli* strain BL21/DE3-LysS, a large amount of recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The cell lysate (obtained after lysis of cells into 20 mM Tris, pH 7.5, and 8 M urea) was applied to an Ni^{2+}-NTA-Agarose column (Qiagen, Hilden, Germany). The column was extensively washed with 20 mM HEPES, pH 6.3, 8 M urea, and 20 mM imidazole, and the histidine-tagged recombinant protein was eluted with the same buffer containing 250 mM imidazole. The purified protein was estimated to be >95% pure as assessed by SDS-PAGE and Coomassie blue staining. This protein was used to immunize New Zealand white rabbits according to a standard protocol. Antibodies were affinity purified on nitrocellulose strips; briefly, a preparative SDS-PAGE of the immunogen was performed and the protein transferred onto nitrocellulose. The relevant band was visualized with Ponceau S and the strip cut out. After the membrane was blocked with low-fat powdered milk, 1 ml of immune serum was incubated at 4°C for 2 h with the blot that was washed with PBS. The bound antibody was eluted with 1 ml of HCl-glycine 0.1 M, pH 2.5, for 5 min and immediately neutralized with 1 M Tris and dialyzed against PBS.

Fractionation, Immunoprecipitation, and Immunoblotting

To obtain cytosol, we suspended HepG2 cells in lysis buffer (20 mM HEPES, pH 6.8, 100 mM CH_3COOK, 5 mM $MgCl_2$, and 8% sucrose), which were then lysed with 20 strokes of a tight-fitting glass potter in the presence of 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and protease inhibitors (Complete™ protease inhibitor cocktail tablets; Boehringer Mannheim, Mannheim, Germany). The postnuclear supernatant of the homogenate was centrifuged at 100,000 × *g* for 60 min at 4°C, snap-frozen in liquid nitrogen, and stored at −80°C. For fractionation experiments, the postnuclear supernatant was subjected to treatment in 4 M urea or in 0.1 M carbonate buffer, pH 11, at 4°C for 30 min before being ultracentrifuged at 100,000 × *g* for 75 min. The pellet and the supernatant fractions were brought to an equal volume with sample buffer and analyzed by SDS-PAGE and immunoblotting. Yeast cytosol was obtained by lysis with glass beads at 4°C in the above lysis buffer and treated similarly to HepG2 cytosol. Whole-yeast extracts were prepared by vortexing cells in hot SDS-PAGE sample buffer in the presence of glass beads.

Yeast and HepG2 cytosol were fractionated onto either AcA34 or Superdex 200 equilibrated in PBS. Each 500-μl fraction was concentrated by acetone precipitation and resuspended in SDS-PAGE sample buffer. The absorbance at 280 nm was used to quantify the total protein concentration in each fraction, and an equal amount of protein from each fraction was loaded onto gels for immunoblotting analysis. Proteins separated by SDS-PAGE were transferred onto 0.45-μm nitrocellulose membranes and saturated for at least 2 h in PBS/3% low-fat milk. Antibodies were diluted into PBS/1% low-fat milk, and incubation was performed overnight at 4°C. After extensive washing with PBS containing 0.05% Tween 20, the blots were incubated for 1 h with HRP-conjugated goat anti-rabbit immunoglobulin (Ig, diluted 1:7500; Amersham) or HRP-conjugated sheep anti-Mouse Ig (diluted 1:3000; Amersham). After extensive washing, blots were revealed by electrochemiluminescence (ECL kit, Amersham).

To immunoprecipitate hSec23A, we grew HepG2 cells in 6-cm diameter Petri dishes to subconfluence and labeled them for 6 h at 37°C with 250 μCi/ml of Trans^{35}S-Label in RPMI medium without methionine. Cells were washed three times with ice-cold PBS containing 1 mM sodium azide and lysed in lysis buffer containing 1% Triton X-100 and protease inhibitors. The lysate was clarified in a microcentrifuge for 15 min at 4°C and the supernatant immunoprecipitated with 10 μl of anti-hSec23Ap antiserum, followed by the addition of protein-A Sepharose. Affinity-purified anti-hSec23Ap antibodies did not immunoprecipitate hSec23Ap efficiently, possibly because high-affinity antibodies were not recovered efficiently during affinity purification.

RNase Protection Assay

Total RNA from cultured cells was purified by the guanidinium acid-phenol method. Probes for the two hSec23 isotypes were synthesized with T3 RNA polymerase in the presence of 50 μCi of ^{32}P-UTP and then purified on an acrylamide-urea gel. Probes had a length of 207 and 282 nucleotides for hSec23A and hSec23B, respectively. The RNAse-protected fragments had a size of 190 nucleotides for the isotype hSec23A and 265 nucleotides for hSec23B. We used a human TATA-binding protein (TBP) probe (a kind gift from V. Steimle, University Medical Center, Geneva, Switzerland) to monitor and calibrate the amount of RNA used in each protection assay (length of the undigested and digested probe: 237 and 161 nucleotides, respectively). Hybridization was performed at 45°C for 16 h in 80% formamide, 40 mM PIPES, pH 6.8, 400 mM NaCl, and 2 mM EDTA in a final volume of 20 μl. Ten micrograms of total RNA were hybridized with 300,000 cpm of both hSec23A and hSec23B antisense probes. In some assays, the TBP probe was used together with the hSec23A and hSec23B probes. Single-stranded RNA was digested with a mix of 1 μg of RNAse A and 20 U of RNAse T1 for 30 min at 37° in 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, and 4 mM EDTA. The reaction was stopped by the addition of 20 μl of 10% SDS and 2.5 μl of 20 μg/ml proteinase K and incubated for an additional 15 min. RNAse-protected fragments were extracted twice with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1) and then precipitated with ethanol. The pellet was washed with 70% ethanol and resuspended in 10 μl of formamide-loading buffer (80% deionized formamide, 1 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). Samples were electrophoresed on a 6% polyacrylamide/urea sequencing gel, and the intensity of each protected fragment was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The ratio of hSec23A over hSec23B was calculated, and the relative amount of each isotype was compared with the amount of TBP.

Immunocytochemistry

Indirect Immunofluorescence. For immunofluorescence microscopy, we fixed monolayers of cells in 4% paraformaldehyde in PBS and then permeabilized the cells by dehydration and rehydration with ethanol. Affinity-purified anti-hSec23A antibodies were incubated for 2 h in a moist chamber, followed by washing with PBS and exposure to FITC-conjugated goat anti-rabbit IgG. Counterstaining was performed by incubation of samples in 0.03% Evans blue before examination with a confocal microscope. As a control, specific antibodies were first adsorbed with 50 μg of the recombinant immunogen for 30 min at room temperature before incubation with cells.

Immunoelectron Microscopy. Fragments of pancreatic tissues from normal rats were fixed with 1% glutaraldehyde in 0.1 M sodium phosphate for 1 h. The tissue was then washed with buffer, infiltrated with sucrose, and processed for cryoultramicrotomy as described (Tokuyasu, 1986). Cells grown in monolayer were resuspended in PBS and fixed as a pellet with 1% glutaraldehyde and processed as described above. Immunolocalization was performed by incubating the sections with the first antibody (dilution 1:4) at room temperature for 1 h. The antibodies were revealed by the protein A-gold method (Roth *et al.*, 1978), and sections were absorption stained with uranyl acetate before examination.

RESULTS

Cloning of Human Sec23 Isoforms

To isolate the full-length cDNA of the mammalian homologue of Sec23p, oligonucleotide primers specific for conserved regions of the yeast and a partial mouse cDNA clone were used to prime PCR amplification of cDNA of mouse and human origin (see MATERIALS AND METHODS). A fragment of the expected size was obtained from both mouse and human cDNA, and the human fragment was used to screen a human B lymphocyte cDNA library. A clone of 3.8 kb was selected and sequenced. It contained an open reading frame of 2298 nucleotides encoding a protein sharing 47.3% identity with yeast Sec23p; this clone was named hSec23A (Figure 1). The search for homologues by using the BLAST algorithm (Altschul *et al.*, 1990) identified the clone EST65274 that shared >80% identity with hSec23A clone. An oligonucleotide discriminating between the two sequences was designed to probe the same library. Sequencing of a 2.8-kb clone confirmed the existence of a second closely related Sec23 homologue. The open reading frame of 2304 bp encoded a protein that shared 84.3% identity with the product of hSec23A and 47.6% identity with the yeast Sec23p (Figure 1). This second clone was named hSec23B. No other significant homologies with published sequences of known function were found in the databases. However, several EST clones or partial cDNA sequences from *Homo sapiens*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* share significant homologies with either hSec23A or hSec23B sequences.

Intracellular Localization

Antibodies against a recombinant N-terminal portion of hSec23A were raised and affinity purified. These antibodies recognized an 85-kDa polypeptide in extracts from the human cell line HepG2 (Figure 2A). The antibodies also cross-reacted with an 85-kDa protein in mouse and rat tissues but did not cross-react with endogenous yeast Sec23p (Figures 2A and 6B). Because of the high sequence homology between the two isoforms, we expected that antibodies generated against the hSec23Ap isoform would cross-react with hSec23Bp. We took advantage of the absence of cross-reactivity with yeast Sec23p to test this hypothesis by transforming the yeast RSY868 with each isoform and probing cells lysates by immunoblotting with affinity-purified anti-hSec23A antibodies. Surprisingly, we could not detect any signal in cells expressing hSec23Bp (Figure 2C). However, a protein of the expected molecular mass was detected when the hSec23B isoform was tagged at its N terminus with the HA epitope and expressed in yeast (Figure 2C). Thus, our affinity-purified antibodies recognized only the hSec23Ap isoform.

Both hSec23 isoforms are predicted to be cytosolic proteins. By subcellular fractionation of postnuclear extracts of HepG2 cells, we found that hSec23Ap partitioned equally between cytosol and membranes in the condition used for cell disruption; however, hSec23Ap remained soluble when lysates were treated with 4 M urea or carbonate extraction at pH 11, suggesting that the particulate fraction represented membrane-bound hSec23Ap (unpublished observations).

In yeast, Sec23p is associated in a complex with another cytosolic protein, Sec24p (Hicke *et al.*, 1992). To determine whether this could be the case in HepG2 cells, we fractionated HepG2 cytosol on a gel filtration column. The peak of hSec23Ap immunoreactivity chromatographed to a position equivalent to a complex of 350 kDa (Figure 3A), whereas none was detected at the expected monomeric molecular mass of 85 kDa. Fractions corresponding to the void volume of the column (not included in Figure 3A) contained a small amount of immunoreactive material corresponding to very large (>2000 kDa) protein aggregates. When probed with yeast anti-Sec23 antiserum, bands at an identical molecular mass in the same fractions were identified, indicating that anti-hSec23A antibodies and anti-yeast Sec23 antiserum recognize the same protein (our unpublished observation). To identify the putative partner(s) of hSec23Ap, HepG2 cells were metabolically labeled, and the postnuclear supernatant was subjected to immunoprecipitation. Two majors bands were immunoprecipitated, one at

Figure 1 (on facing page). Alignment of protein sequences of hSec23Ap, hSec23Bp, Msec23p, and yeast Sec23p (single-letter amino acid code) obtained by translation of the ORF of their respective cDNA clone with the GCG package. Identical residues are boxed. DNA sequences have been submitted to European Molecular Biology Laboratory (EMBL) data library (hSec23A, accession number X97064; hSec23B, accession number X97065).

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hsec23a     1 MTTYLEFIQQNEERDGVRFSWNVWPSSRLEATRMVVPVAALFTPLKERPDLPPIQYEPVL
hsec23b     1 MATYLEFIQQNEERDGVRFSWNVWPSSRLEATRMVVPLACLLTPLKERPDLPPVQYEPVL
msec23p     1 MTTYLEFIQQNEERDGVRFSWNVWPSSRLEATRMVVPVAALFTPLKERPDLPPIQYEPVL
ysec23p     1 ....MDF.ETNEDINGVRFTWNVFPSTRSDANSNVVPVGCLYTPLKEYDELNVAPYNPVV

hsec23a    61 CSRTTCRAVLNPLCQVDYRAKLWACNFCYQRNQFPPSYAGISELNQPAELLPQFSSIEYV
hsec23b    61 CSRPTCKAVLNPLCQVDYRAKLWACNFCFQRNQFPPAYGGISEVNQPAELMPQFSTIEYV
msec23p    61 CSRTTCRAVLNPLCQVDYRAT.RACNFV..PRSVPPTYSGISELNQPREFY..LVSDEYV
ysec23p    56 CSGPHCKSILNPYCVIDPRNSSWSCPICNSRNHLPPQYTNLSQENMPLEL..QSTTIEYI

hsec23a   121 VLRGPQMPLIFLYVVDTCMEDEDLQALKESMQMSLSLLPPTALVGLITFGRMVQVHELGC
hsec23b   121 IQRGAQSPLIFLYVVDTCLEEDDLQALKESLQMSLSLLPPDALVGLITFGRMVQVHELSC
msec23p   116 VLRGPQMPFIFLYVVDTCIEDEDLQALKESMQTTFSLFPPTALVGLITFGRIVQVHELGC
ysec23p   114 TNKPVTVPPIFFFVVDLTSETENLDSLKESIITSLSLLPPNALIGLITYGNVVQLHDLSS

hsec23a   181 EGISKSYVFRGTKDLSAKQLQEMLGLSK..VPVTQATRGPQVQQ...PPPSNRFLQPVQK
hsec23b   181 EGISKSYVFRGTKDLTAKQIQDMLGLTKPAMPMQQARPAQPQEH...PFASSRFLQPVHK
msec23p   176 EH.SKSYVFRGTKDLSAKQLQEMLGLSK..VPVTQATSRSSGTA...TATFNRFLQPVQK
ysec23p   174 ETIDRCNVFRGDREYQLEALTEMLTGQKPTGPGGAASHLPNAMNKVTPFSLNRFFLPLEQ

hsec23a   236 IDMNLTDLLGELQRDPWPVPQGKRPLRSSGVALSIAVGLLECT.....FPNTGARIMMFI
hsec23b   238 IDMNLTDLLGELQRDPWPVTQGKRPLRSTGVALSIAVGLLEGT.....FPNTGARIMLFT
msec23p   230 IDMNLTDLLGELQRDPWPVPQGKRPLRPSGVALSIAVGLLECTPQHWCSDHDVHRCLL..
ysec23p   234 VEFKLNQLLENLSPDQWSVPAGHRPLRATGSALNIASLLLQG.....CYKNIPARIILFA

hsec23a   291 GGPATQGPGMVVGDELKTPIRSWHDIDKDNAKYVKKGTKHFEALANRAATTGHVIDIYAC
hsec23b   293 GGPPTQGPGMVVGDELKIPIRSWHDIEKDNARFMKKATKHYEMLANRTAANGHCIDIYAC
msec23p   288 .....PGPGMVVGDELKTPMRSWHDIEKDNPNMLKRELSILKRWLIELLQRGMSL.ISTP
ysec23p   289 SGPGTVAPGLIVNSELKDPLRSHHDIDSDHAQHYKKACKFYNQIAQRVAANGHTVDIFAG

hsec23a   351 ALDQTGLLEMKCCPNLTGGYMVMGDSFNTSLFKQTFQRVFTKDMHGQFKMGFGGTLEIKT
hsec23b   353 ALDQTGLLEMKCCANLTGGYMVMGDSFNTSLFKQTFQRIFTKDFNGDFRMAFGATLDVKT
msec23p   342 VLDQTGLLEMKCCPNLTGGYMVMGDSFNTSLFKQTFQRVFTKDIHGQFKMGFGGTLEIKT
ysec23p   349 CYDQIGMSEMKQLTDSTGGVLLLTDAFSTAIFKQSYLRLFAKDEEGYLKMAFNGNMAVKT

hsec23a   411 SREIKISGAIGPCVSLNSKGP.CVSENEIGTGGTCQWKICGLSPTTTLAIYFEVVN...Q
hsec23b   413 SRELKIAGAIGPCVSLNVKGP.CVSENELGVGGTSQWKICGLDPTSTLGIYFEVVN...Q
msec23p   402 SREIKISGAIGPCVLLIQKDL.ACLKMRLEQEALVSGK.SVASPTTTLAIYFEVVN...Q
ysec23p   409 SKDLKVQGLIGHASAVKKTDANNISESEIGIGATSTWKMASLSPYHSYAIFFEIANTAAN

hsec23a   467 HNAPIPQGGRGA......IQFVTQYQHSSGQRRIRVTTIARNWADAQTQIQNIAASFDQE
hsec23b   469 HNTPIPQGGRGA......IQFVTHYQQSSTQRRIRVTTIARNWADVQSQLRHIEAAFDQE
msec23p   457 HNAPIPQGGRGA......VQFVTQYQHSSGQRRIRVTTIARNWADAQTQIQNIARSFDQE
ysec23p   469 SNPMMSAPGSADRPHLAYTQFITTYQHSSGTNRIRVTTVANQLLPFGT..PAIAASFDQE

hsec23a   521 AAAILMARLAIYRAETEEGPDVLRWLDRQLIRLCQKFGEYHKDDPSSFRFSETFSLYPQF
hsec23b   523 AAAVLMARLGVFRAESEEGPDVLRWLDRQLIRLCQKFGQYNKEDPTSFRLSDSFSLYPQF
msec23p   511 AAPILMARLAIYRAETEEGPDVLRWLDRQLIRLCQKFGEYHKDDPNSFRFSETFSLYPQF
ysec23p   527 AAAVLMARIAVHKAETDDGADVIRWLDRTLIKLCQKYADYNKDDPQSFRLAPNFSLYPQF

hsec23a   581 MFHLRRSSFLQVFNNSPDESSYYRHHFMRQDLTQSLIMIQPILYAYSFSGPPEPVLLDSS
hsec23b   583 MFHLRRSPFLQVFNNSPDESSYYRHHFARQDLTQSLIMIQPILYSYSFHGPPEPVLLDSS
msec23p   571 MFHLRRSPFLQVFNNSPDESSYYRHHFMRQDLTQSLIMIQPILYAYSFSGR.........
ysec23p   587 TYYLRRSQFLSVFNNSPDETAFYRHIFTREDTTNSLIMIQPTLTSFSMEDDPQPVLLDSI

hsec23a   641 SILADRILLMDTFFQILIYHGETIAQWRKSGYQDMPEYENFRHLLQAPVDDAQEILHSRF
hsec23b   643 SILADRILLMDTFFQIVIYLGETIAQWRKAGYQDMPEYENFKHLLQAPLDDAQEILQARF
msec23p       ............................................................
ysec23p   647 SVKPNTILLLDTFFFILIYHGEQIAQWRKAGYQDDPQYADFKALLEEPKLEAAELLVDRF

hsec23a   701 PMPRYIDTEHGGSQARFLLSKVNPSQTHNNMYAWGQESGAPILTDDVSLQVFMDHLKKLA
hsec23b   703 PMPRYINTEHGGSQARFLLSKVNPSQTHNNLYAWGQETGAPILTDDVSLQVFMDHLKKLA
msec23p       ............................................................
ysec23p   707 PLPRFIDTEAGGSQARFLLSKLNPSDNYQDM...ARGGSTIVLTDDVSLQNFMTHLQQVA

hsec23a   761 VSSAA
hsec23b   763 VSSAC
msec23p       .....
ysec23p   764 VSGQA
```

Figure 1.

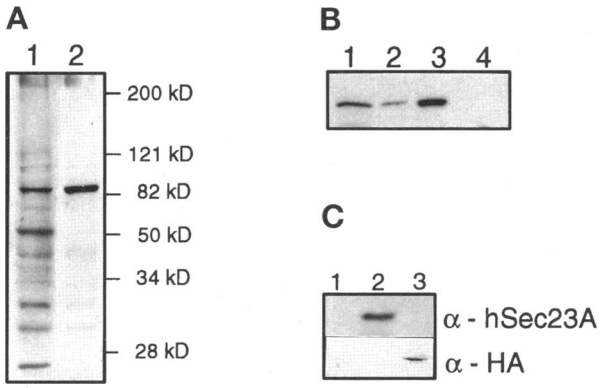

Figure 2. Immunoblotting of total cell extracts separated on a 10% SDS-PAGE and probed with anti-hSec23Ap. (A) HepG2 extract probed with antiserum (lane 1) or affinity-purified (lane 2) antibodies against hSec23Ap. (B) Equivalent amounts of total cell extracts probed with affinity-purified anti-hSec23Ap; lane 1, HepG2; lane 2, rat islets of Langerhans; lane 3, mouse liver; lane 4, yeast RSY620. (C) Yeast cytosol expressing the mock plasmid (lane 1), hSec23A (lane 2), and hSec23B-HA (lane 3) probed with either affinity-purified anti-hSec23Ap antibodies or monoclonal anti-HA 12CA5.

the expected molecular mass of hSec23Ap and another larger band with an apparent molecular mass on SDS-PAGE of ~110 kDa (Figure 3B).

Affinity-purified anti-hSec23Ap antibodies were used to analyze by immunocytochemistry the intracellular distribution of hSec23Ap. Immunofluorescence microscopy showed that hSec23Ap was concentrated at the periphery of the nucleus in a punctiform pattern in HepG2 and NRK cells (Figure 4, A and C). hSec23Ap distribution was not altered upon treatment with 5 μg/ml of brefeldin A (BFA) for 30 min (Compare Figure 4, C and D). By immunoelectron microscopy performed on rat pancreatic acinar cells as well as on HepG2 cells, we detected hSec23Ap restricted to the tubular extensions of the transitional ER and its associated vesicles (Figure 5, A and B). No significant labeling of other structures was observed. This distribution corresponded to that observed with antibodies directed against the yeast protein (Orci *et al.*, 1991). Both for immunofluorescence and immunoelectron microscopy, the efficiency of BFA treatment was assessed by monitoring the change in intracellular distribution of β-COP.

Complementation of the Yeast Temperature-sensitive Mutant sec23-1 Defect by the Mammalian Homologues

Similarities in the deduced amino acid sequence of both forms of mammalian Sec23p (hSec23Ap and hSec23Bp) with yeast Sec23p suggested that the mammalian proteins could be functional homologues of the yeast gene product. This hypothesis was verified by testing whether the hSec23A and hSec23B isoforms could correct the temperature sensitivity of the yeast mutant strain *sec23-1* in vivo. hSec23A and hSec23B cDNAs were subcloned into a multicopy yeast expression vector under control of the regulatable *GAL1* promoter and introduced into *sec23-1* ts strain RSY868. The untransformed *sec23-1* mutant fails to grow above 32°C (Kaiser and Schekman, 1990). At the permissive temperature (26°C) cells transformed with *GAL1*-hSec23A grew on glucose, which repressed hSec23A expression, as well as on 2% galactose/0.2% glucose, which induced its expression. Preliminary experiments showed that cells harboring either *GAL1*-hSec23A or *GAL1*-hSec23B constructs grew at a slower rate when plated on 2% galactose, whereas the addition of a small amount of glucose enabled them to grow as wild-type cells. Thus we chose 2% galactose/0.2% glucose as the induction medium thereafter. At the restrictive temperature of 34°C, only cells containing hSec23A plated on 2% galactose/0.2% glucose sustained growth (Figure 6A). Complementation by the human gene product was also observed at 37°C (unpublished observations). Growth of the *sec23-1* strain at the restrictive temperature was correlated with the expression of the human protein, as assessed by immunoblotting with affinity-purified hSec23A antibodies (Figure 6B).

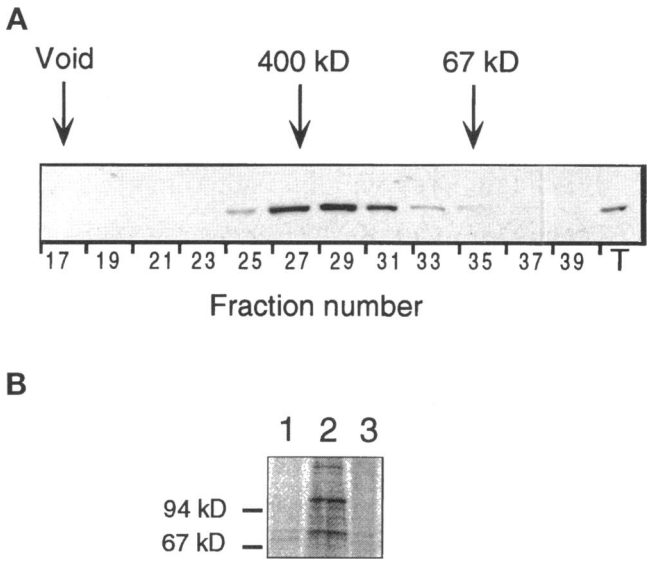

Figure 3. (A) Fractionation of HepG2 cytosol by gel filtration on an AcA34 column. Cytosol (3 mg) was loaded onto the column, and the collected fractions were electrophoresed on a 10% SDS-polyacrylamide gel. The position of hSec23Ap was detected by immunoblotting; T, unfractionated cytosol. Vertical arrows denote the elution of standards used to calibrate the column, and void volume was estimated with blue dextran. (B) Immunoprecipitation of HepG2 extract labeled with ^{35}S-methionine and immunoprecipitated with none (lane 1), anti-hSec23A serum (lane 2), or preimmune serum (lane 3).

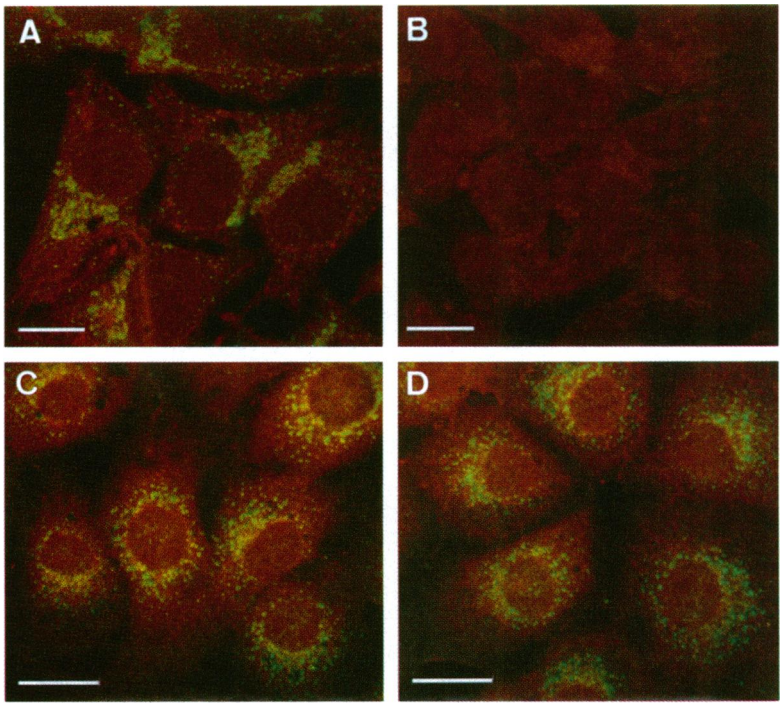

Figure 4. Localization of hSec23Ap by immunofluorescence. (A) hSec23Ap appears concentrated in a juxtanuclear region in HepG2 cells and displays a characteristic punctate pattern. (B) HepG2 cells incubated with antibodies preadsorbed with 50 μg of the recombinant N-terminal fragment of hSec23A. In NRK cells (C), a similar distribution is found, which is not modified by BFA treatment (D). Bar, 20 μm.

When tested under the same conditions, the second isotype hSec23B did not display any complementation activity (unpublished results). A defect in transcription of the cloned gene by the *GAL1*-regulated plasmid can be ruled out, because large quantities of mRNA were produced by cells containing the hSec23B isoform, as detected by RNAse protection assay. Moreover, translation of the human gene product was normal, because we detected an HA-tagged hSec23B form by immunoblotting a yeast cell lysate with monoclonal antibodies against the HA epitope (Figure 2C). To investigate whether the levels of overproduction of hSec23Bp were so severe as to have a toxic effect, we lowered the induced level of the protein by increasing the amount of glucose in the 2% galactose-containing medium from 0.1 to 0.5%. Neither hSec23B nor the HA-tagged hSec23B isoform complemented the growth defect of the *sec23-1* strain at the restrictive temperature (our unpublished observations).

Another possibility to explain the lack of complementation of the hSec23B isoform could be its incapacity to form a stable and/or functional complex with endogenous yeast Sec24p (Hicke *et al.*, 1992). We compared the ability of each isoform to form a complex in yeast by fractionating cytosol from cells expressing either hSec23A or HA-tagged hSec23B and analyzing each fraction by immunoblotting. Samples of equal protein amounts showed that the majority of hSec23Ap migrated at the position of the 350-kDa complex, whereas the HA-tagged hSec23B isoform distributed into fractions ranging in size from 80 to 350 kDa (Figure 7). Aliquots of equal volume analyzed by immunoblotting showed some hSec23Ap migrating at the position expected of a monomeric species, possibly the result of overproduction of the protein under these growth conditions.

Tissue Expression

The existence of two isoforms of hSec23p raised the possibility that these proteins may exhibit functional differences. As a first step toward determining the role of each isoform, we measured the respective tissue expression level of mRNA encoding both forms of the protein by RNAse protection assays. Isoform-specific probes to the 3′-end of each ORF were such that the protected fragments were of different sizes, and mRNA extracted from human cell cultures and tissues was analyzed with both probes simultaneously (Figure 8). The ratio between the two probes was quantified, and their respective abundance in each tissue was compared in relation to the constitutively expressed human TATA-binding protein (TBP) mRNA (Steimle *et al.*, 1994). Both hSec23A and hSec23B isoforms were expressed in all tissues and cell lines examined; however, the ratio of hSec23A to hSec23B varied greatly (Figure 9A). HepG2 cells expressed hSec23A highly, in contrast to Raji cells that showed the opposite; most of the other tissues analyzed had a less pronounced difference in the hSec23A to hSec23B ratio. The relative abundance of both isoforms ranged from two times the amount of the housekeeping gene TBP in

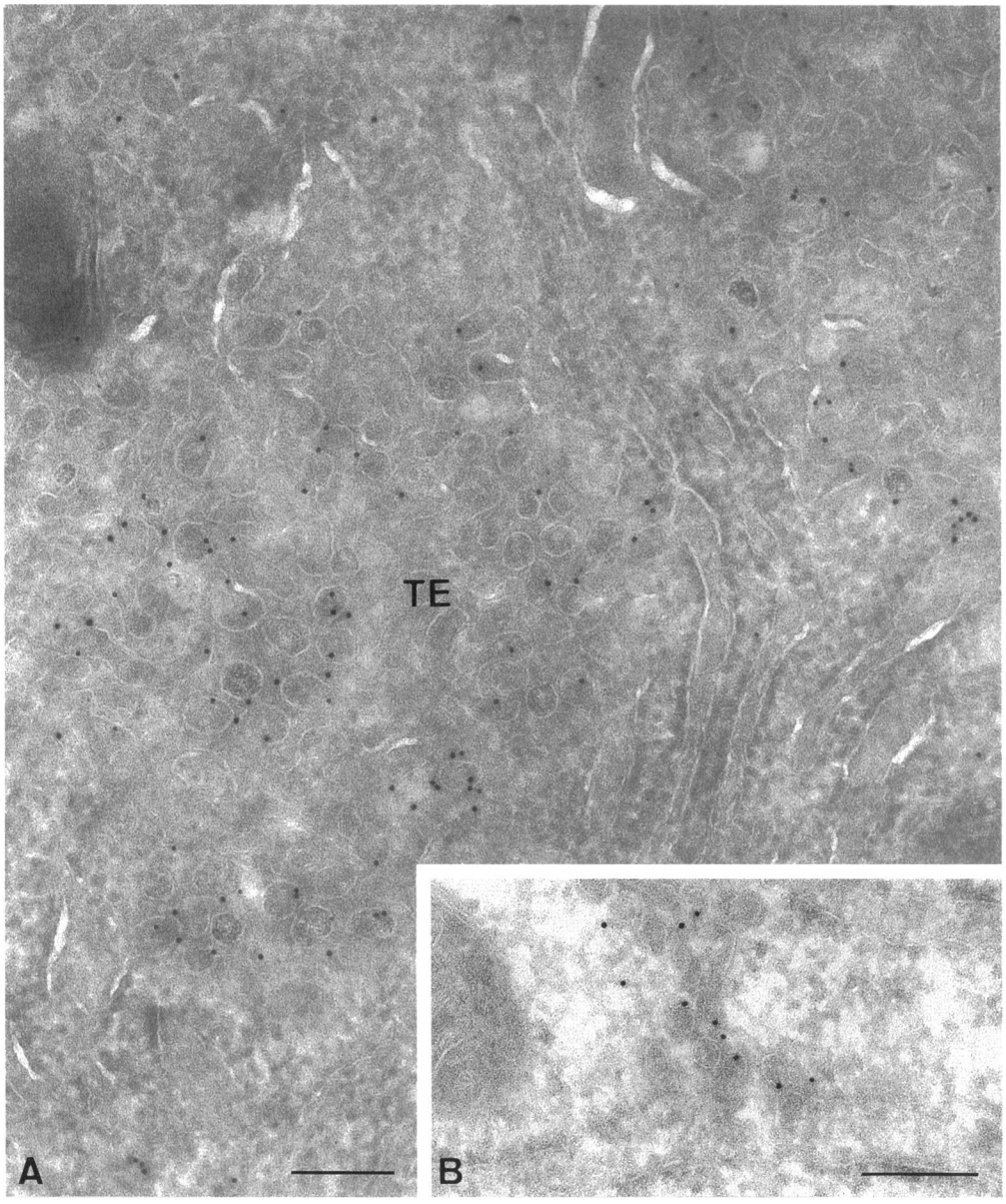

Figure 5. Distribution of hSec23Ap in rat pancreatic acinar cells by electron microscope immunolabeling. In both control and BFA-treated cells (A), hSec23Ap is restricted to the transitional area of the ER (TE) and its associated transfer vesicles. (B) Note the specific labeling of transport vesicles in HepG2 cells. Size of gold particles, 10 nm; bar, 0.2 μm.

Raji cells to 15 times the TBP amount in HepG2 cells (Figure 9B).

DISCUSSION

In this report we describe the molecular cloning and characterization of two human homologues of the yeast COPII component *SEC23*. The existence of a mammalian counterpart of yeast Sec23p was suspected on the basis of the observation that antibodies directed against the yeast protein cross-react with an 84-kDa protein in mouse liver and pancreas extracts and labeled regions of the mammalian ER involved in transport vesicle formation (Orci *et al.*, 1991). Moreover, a mouse cDNA encoding a protein with similarities to yeast Sec23p was described (Wadhawa *et al.*, 1993). However, this mouse clone predicted a protein of only 64.7 kDa, lacking the 200 C-terminal amino acids of the yeast Sec23p. Examination of the mouse sequence revealed a potential frame shift leading to an early termination codon. On the basis of homologies between the partial mouse clone and the yeast sequence, we isolated two related cDNAs from a human library. Sequencing analysis and comparison with the mouse Msec23 confirmed our assumption that the latter is probably an incomplete version of the bona fide mouse *SEC23* gene. A search of the

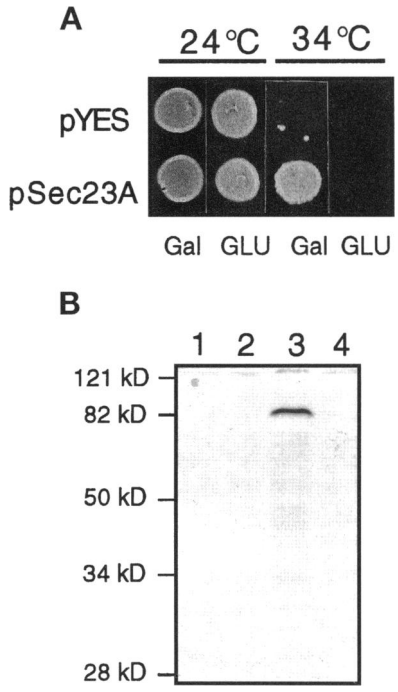

Figure 6. Effect of the human hSec32Ap protein in the temperature-sensitive *sec23-1* mutant strain. (A) hSec23Ap was expressed in the *sec23-1* strain on the episomal yeast expression vector pYES2 under the *GAL1* promoter and incubated at permissive (24°C) or restrictive (34°C) temperatures on either glucose or galactose. The human gene complements the temperature sensitivity of the *sec23-1* mutant strain. (B) This complementation is correlated with the expression of the human protein in cells grown at a restrictive temperature, as assessed by Western blotting of total cell extracts with affinity-purified anti-hSec23Ap antibodies (see MATERIALS AND METHODS and Figure 3). Lanes 1 and 2, pYES2; lanes 3 and 4, pSec23A; lanes 1 and 3, galactose; lanes 2 and 4, glucose. Antibodies do not cross-react with endogenous yeast Sec23p (lanes 3 and 4).

databases with the predicted full length of both proteins did not identify any significant homologies with proteins of known function. However, the finding of several EST clones from *C. elegans* and *A. thaliana* with significant homologies to both human Sec23 isoforms renders likely the existence of similar isoforms in these organisms.

The hSec23A isoform complemented the growth temperature sensitivity of the yeast mutant *sec23-1* up to 37°C, an effect that was conditional to the expression of the human protein as assessed by immunoblotting. This stringent assay insured that hSec23A is indeed a functional homologue of the yeast Sec23p. Yeast Sec23p is incorporated into a complex, along with Sec24p, to promote vesicle budding (Hicke *et al.*, 1992). Likewise, we expected that the complementing human gene product would associate into a complex with Sec24p in yeast cytosol. In agreement with this predicted result, we showed that a large proportion of hSec23Ap expressed in yeast chromatographed on a gel filtration column as a complex. In HepG2 cytosol, hSec23Ap also isolated as a complex of ~350 kDa, and a polypeptide of ~110 kDa was coimmunoprecipitated with hSec23Ap. This polypeptide is likely to be the mammalian homologue of the yeast Sec24p.

Although the degree of homology between the two isoforms exceeds 80% and the two proteins share 48% homology with the yeast Sec23p, the hSec23B isoform did not complement the temperature-sensitive *sec23-1* strain. We showed that this was not due to a defect in transcription of the construct, because equivalent amounts of both hSec23A and hSec23B transcripts were detected in an RNAse protection assay. Affinity-purified antibodies raised against the N-terminal portion of hSec23Ap failed to recognize a band at the expected molecular weight in yeast expressing hSec23B despite a high level of specific mRNA expression, raising the possibility that translation of the gene product was impaired or that antibodies did not cross-react with the hSec23Bp isoform. Using a HA-tagged form of hSec23Bp, we showed that the protein was indeed synthesized in yeast and that the antibodies generated were specific for the hSec23A isoform. An inappropriate level of expression of the mammalian protein in yeast could result in large amounts of monomeric hSec23B in the cytosol, a condition known to inhibit cell growth (Yoshihisa and Schekman, unpub-

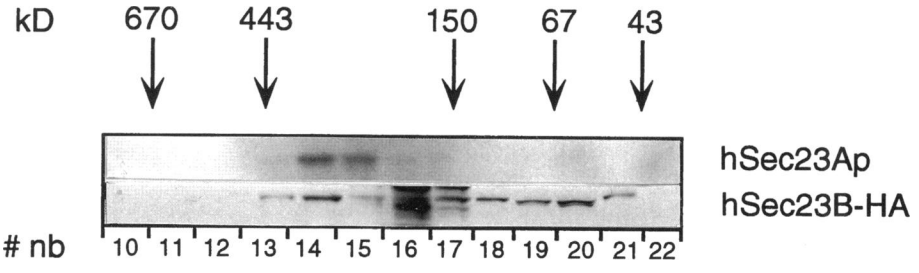

Figure 7. Complex formation by hSec23Ap and hSec23B-HAp in yeast cytosol. RSY868 was transformed with either hSec23A or hSec23B-HA and grown on 2% galactose/0.2% glucose to an OD_{600} of 1.0. Cytosol was obtained by glass bead lysis of cells and fractioned on Superdex 200. An equivalent amount of protein from each fraction was loaded onto a 9% polyacryamide gel, and fractions were analyzed by immunoblotting with either anti-hSec23Ap antibodies or monoclonal anti-HA 12CA5. Bands above and below the HA-tagged species in fractions 16 and 17 are also present in similar fractions from cytosol expressing the hSec23Ap isoform, thus representing yeast proteins cross-reacting with anti-HA antibodies. Arrows indicate the elution position of markers used to calibrate the column.

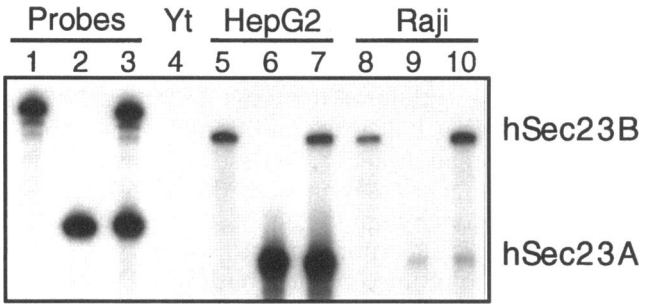

Figure 8. Estimation of mRNA expression level by RNAse protection assay. Total mRNA (5 or 10 mg) from either human HepG2 hepatoma cells or Raji B lymphocytes was hybridized with labeled hSec23A and/or hSec23B-specific probes. After digestion with RNAses, the probes were separated on an acrylamide-urea gel, and the amount of protected probe was quantified by fluorography. Lanes 1–3, undigested probes for hSec23B, hSec23A, and both together, respectively; lane 4, both probes with control yeast tRNA; lanes 5–7, HepG2 mRNA with hSec23B, hSec23A, and both probes, respectively; lanes 8 and 9, Raji mRNA with hSec23B, hSec23A, and both probes, respectively.

lished observations). However, this is unlikely to interfere with complementation, because modulation of the *GAL1* promoter used to express hSec23B produced no condition in which either hSec23B or HA-tagged hSec23B isoform allowed growth at the restrictive temperature. We cannot exclude the fact that the presence of the amino-terminal tag interferes with hSec23B function; however, the untagged hSec23B also did not complement the *sec23-1* mutation. Another possibility suggested by gel filtration experiments is that the hSec23B isoform cannot engage in a complex with yeast Sec24p.

By immunofluorescence and immunoelectron microscopy, we localized hSec23A to the transitional ER, a specialized region of the cell that most likely represents a major site of exit for proteins leaving the ER en route to the Golgi apparatus. A comparable distribution has been observed previously with anti-yeast Sec23p antibodies (Orci *et al.*, 1991) and is also similar to the other mammalian COPII component SEC13Rp and Sar1p (Kuge *et al.*, 1994; Shaywitz *et al.*, 1995). BFA does not induce redistribution of hSec23A, which is also the case for Sec13Rp (Orci *et al.*, 1994; Shaywitz *et al.*, 1995). This feature distinguishes it from COPI components such as β-COP, the distribution of which is altered upon BFA treatment. The subcellular localization of hSec23A and its functional analysis in yeast provide strong evidence that hSec23A is part of the mammalian COPII complex, which plays an essential role in the formation of transport vesicles from the ER.

The existence of two isoforms of hSec23 is not an exception among mammalian proteins related to the secretory pathway; isoforms of Sar1, Rab1, and SNAP have been described (Shen *et al.*, 1993; Whiteheart *et al.*, 1993; Kuge *et al.*, 1994). No isoform-specific functional analysis of Sar1 or Rab1 has been reported; however, the Sar1b isoform is found essentially in liver and skeletal muscles, whereas the Sar1a transcript is expressed in most tissues examined (Shen *et al.*, 1993). β-SNAP is restricted to the brain (Whiteheart *et al.*, 1993). By contrast, hSec23A and hSec23B mRNA

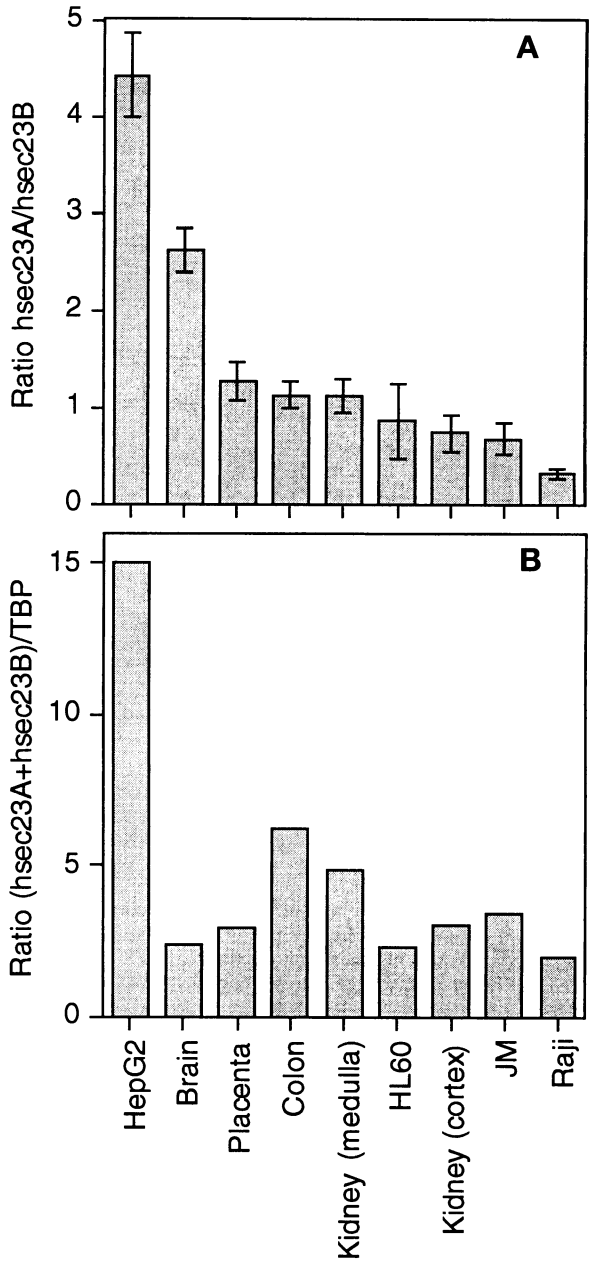

Figure 9. Quantification of the mRNA expression levels of hSec23A and hSec23B in different human tissues and cell lines. (A) Relative expression of hSec23A versus hSec23B mRNAs; average of 2–5 experiments; error bar, ± SEM. (B) Relative abundance of both hSec23A and hSec23B isotypes as compared with TBP mRNA expression (data representative of two experiments).

are expressed simultaneously in all the human tissues and cell cultures examined. It is possible that the hSec23B isoform plays a role similar to hSec23A in COPII-dependent vesicle formation. If so, the COPII coat may incorporate a variable proportion of both hSec23 isoforms or exclude one of them. In either case, selective mechanisms may exist that dictate the ratio of hSec23Ap to hSec23Bp incorporated into a vesicle. Perhaps the hSec23 isoforms are related to cargo selection, bringing different cargo or adapter molecules together in a common vesicle or segregating distinct sets of cargo molecules into different vesicles. Alternatively, the two isoforms may have different functions and thus be targeted to different limbs of the secretory pathway. To answer these questions, a precise intracellular localization of hSec23Bp is needed, which will require the development of an isoform-specific antiserum.

ACKNOWLEDGMENTS

We thank Christine Maeder-Garavaglia for her skillful technical assistance and Pierre-Alain Menoud for providing human mRNAs and for hints on the RNAse protection assay. We are grateful to S. Bednarek and M. Latterich for comments on this manuscript. This work has been supported by the Swiss Science National Foundation (L.O. and J.-L.C.), the Human Frontier Science Program (L.O. and R.S.), and the Howard Hughes Medical Institute (R.S.).

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